1244210

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 01, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/493,389*
FILING DATE: *August 06, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/25477*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

PROVISIONAL APPLICATION FOR PATENT
COVER SHEET

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **A METHOD WITH INCREASED YIELD FOR PRODUCTION OF POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES USING HYDRAZIDE CHEMISTRY**

Name of First Inventor: Robert Lee Che-Hung
Residence Address: 116 Amberleigh Drive, Silver Spring, MD 20905

Name of Second Inventor: Carl E. Frasch
Residence Address: 13100 Burlwood Drive, Rockville, MD 20853 .

Enclosed are:

(X) Specification in 16 pages.

(X) 5 sheets of drawings.

(X) A check in the amount of $160 to cover the filing fee is enclosed.

(X) A return prepaid postcard.

(X) The Commissioner is hereby authorized to charge any additional fees which may be required, now or in the future, or credit any overpayment to Account No. 11-1410.

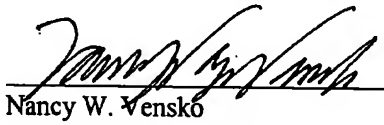
Was this invention made by an agency of the United States Government or under a contract with an agency of the United States Government?

(X) Yes. The name of the U.S. Government agency is: National Institutes of Health.

(X) Please send correspondence to:

Nancy W. Vensko
Knobbe, Martens, Olson & Bear, LLP
2040 Main Street, 14th Floor
Irvine, CA 92614

Respectfully submitted,



Nancy W. Vensko
Registration No. 36,298
Customer No. 20,995
(805) 547-5580

O:\DOCS\NWV\NWV-8587.DOC:cbm
080603

Knobbe Martens Olson & Bear LLP
Intellectual Property Law

1114 Marsh Street
San Luis Obispo CA 93401
Tel 805-547-5580
Fax 805-547-5590

www.kmob.com

Nancy W. Vensko
805-547-5585
nvensko@kmob.com

MAIL STOP PROVISIONAL PATENT APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Attorney Docket No.	:	NIH275.001PR
Applicant(s)	:	Robert Lee Che-Hung and Carl E. Frasch
For	:	A METHOD WITH INCREASED YIELD FOR PRODUCTION OF POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES USING HYDRAZIDE CHEMISTRY
Attorney	:	Nancy W. Vensko
"Express Mail" Mailing Label No.	:	EV 318985198 US
Date of Deposit	:	August 6, 2003

I hereby certify that the accompanying

Transmittal letter; specification in 16 pages; 5 sheets of drawings; Check for Filing Fee; Return Prepaid Postcard

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and are addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Carol B. Miller

Carol B. Miller

O:\DOCS\NWV\NWV-8589.DOC:cbm
080603

A method with increased yield for production of polysaccharide-protein conjugate vaccines using hydrazide chemistry

ABSTRACT

Current methods for synthesis and manufacturing of polysaccharide-protein conjugate vaccines employ conjugation reactions with low efficiency (about 20%). This means that up to 80% of the added activated polysaccharide (PS) is lost. In addition, inclusion of a chromatographic process for purification of the conjugates from unconjugated PS is required. The present invention utilizes the characteristic chemical property of hydrazide groups on one reactant to react with aldehyde groups or cyanate esters on the other reactant with an improved conjugate yield (at least 60%). With this conjugation efficiency the leftover unconjugated protein and polysaccharide ($\leq$35%) would not need to be removed and thus the purification process of the conjugate product can be limited to diafiltration to remove the by-products of small molecules. The new conjugation reaction can be carried out within one or two days with reactant concentrations between 1 and 25 mg/mL at PS/protein ratios from 1:2 to 3:1, at temperatures between 4 and 40°C, and in a pH range of 5.5 to 7.4, optimal conditions varying from PS to PS. Therefore, this invention can reduce the cost of conjugate vaccine manufacture.

INTRODUCTION

Bacterial polysaccharides (PSs) are T-independent antigens inducing short-term immunity in older children and adults but not in young infants. PS-conjugate vaccines are polysaccharide-protein hybrids formed by the covalent attachment of a protein often through its amino acid groups to a chemically modified, or "activated" PS. The chemical modification of the PS is required because native bacterial PSs can not be chemically linked to a protein without first undergoing some chemical modification (activation). Attachment to the protein provides a number of T cell epitopes. These T cell epitopes interact with CD4 helper T cells, greatly facilitating an antibody response to the attached polysaccharide. The T helper cell dependent response to a conjugate results in both serum IgG antibodies and memory B cells, even in infants. Additionally, the immunogenicity of the PS-conjugate, in contrast to the native PS, does not depend upon the size of the conjugated PS; conjugates prepared with either PS or oligosaccharides may have similar immunogenicity.

The Haemophilus influenzae type b (Hib) conjugate vaccines represented the first PS-protein conjugate vaccines produced for clinical use. Robbins and his colleagues in 1980 first utilized the biotechnological process of chemically attaching saccharides to protein carriers developed 50 years [1, 2]. There are now four different Hib conjugate vaccines licensed in the United States, all different, having their own physical, chemical and immunological characteristics (see Table 1). A detailed review of the conjugation chemistry and quality control used in these vaccines has been published [5].

The first commercial conjugate, PRP-D, consisted of partially size-reduced Hib PS attached through a six-carbon spacer, adipic hydrazide (AH), to diphtheria toxoid using the procedure of Schneerson et al.[2]. By this method, the AH derivative of diphtheria toxoid was obtained by reaction with adipic acid dihydrazide (ADH) in the presence of EDC. The Hib PS was then activated through creating cyanate esters on free hydroxyls using CNBr. The activated PS was

then conjugated to the AH-toxoid, but there the process created an unstable linkage and had solubility problems.

The Robbins conjugation chemistry was later modified and the ADH spacer was added first to the polysaccharide, which was then conjugated to the purified protein [6, 7]. This change improved conjugation efficiency and product solubility. The vaccine PRP-T utilized the improved chemistry to covalently link Hib polysaccharide to tetanus toxoid (Table 1).

The PRP-CRM vaccine (HbOC) does not contain Hib PS, but oligosaccharides of about 20 repeat units derived by periodate oxidation of the glycol functionality in the ribitiol moiety. The oxidized oligosaccharides are then attached directly to a nontoxic mutant form of diphtheria toxin known as CRM_{197} as pioneered by Anderson et al.[3]. In this conjugation method the ratio of oligosaccharide to protein was found to be critical for optimal antibody response [5, 8].

Compared to the other Hib conjugate vaccines, PRP-OMPC has a number of unique properties. The protein carrier is not a component of the DTP vaccine, but consists of lipopolysaccharide-depleted meningococcal outer membrane vesicles attached to size-reduced Hib PS through a thioether linkage [4, 5]. In this process separate linkers are attached to both the protein and Hib polysaccharide, followed by fusion of the linkers.

Another group of bacterial polysaccharides to be worked upon for production of conjugates are those from the meningococcus. There are a number of approaches that have been used for activation of the meningococcal PS and for conjugation (Table 2). Each mode of activation has the potential to alter important epitopes, even when relatively few sites are activated on the PS molecule. Periodate activation of the group C meningococcal PS, for example, results in chain breakage generating smaller saccharide units with terminal aldehyde groups that may be linked to the protein via reductive amination [14].

Initial studies on production and optimization of Meningococcal group C conjugates were reported by Beuvery [11, 16, 17] and Jennings [9] well before commercialization of the Hib conjugates. Two differing conjugation methodologies were reported by these investigators for chemically linking the group C PS to a protein carrier [9,16]. The first approach used partially depolymerized PS which was activated by creation of terminal aldehyde groups through periodate oxidation (method 1 in Table 2). The reactive aldehydes then combined through reductive amination with free amino groups on the protein, mostly lysines, in the presence of sodium cyanoborohydride [9]. By this method, activation occurred at one specific site on the group C PS. The second approach utilized the carbodiimide reaction (method 2 in Table 2) to covalently link carboxylic groups in the high molecular weight PS to lysine amino groups on the carrier protein. By this method the activation sites were more random, compared to periodate activation.

Group C meningococcal conjugates prepared by these two methods have been evaluated in animals [10, 17]. Importantly, they stimulated both T cell independent and T cell dependent responses upon initial immunization [17]. The PS must, however, be covalently linked to the carrier protein to induce a T cell dependent antibody response.

The first group A and group C meningococcal conjugates to be used in clinical trials were prepared by Chiron Vaccines and reported in 1992 (method 3 in Table 2) [13]. The conjugation method they developed was based upon selective terminal group activation of small oligosaccharides produced by mild acid hydrolysis and then coupling to a protein through a hydrocarbon spacer. They used the non-toxic mutant of diphtheria toxin, CRM 197, as the protein carrier. To activate the oligosaccharides for conjugation an amino group was added to the end of the oligosaccharide, which was then reacted with the N-hydroxysuccinimide diester of adipic acid to create an active ester. This active ester was then covalently bound to lysine amino groups in the CRM 197 protein creating the conjugate.

Preparation of PS-protein conjugate vaccines has been generally carried out till now using amino groups on the protein (i.e. amino group attaching to a carbon atom) principally from lysine residues, reacting these to functional groups on activated PSs such as aldehyde groups or cyanate groups. The efficiency of the reaction is low, typically about 20% [18] and thus necessitating use of further purification methods to separate the conjugate from unreacted PS. There are a number of possible reasons for such low yield. Firstly, the epsilon amino group of lysine (pKa = 10.5) [19] has low reactivity at the conjugation condition (pH 5.5-7.4). Secondly, most conjugation methods employed toxoids as the carrier protein, which is derived from a toxin by detoxification with formaldehyde, which combines with the amino groups, leaving limited numbers of amino groups available for conjugation. Thirdly, reduced solubility of the resulting protein-PS conjugate due to consumption of hydrophilic groups during conjugation.

To overcome these problems, we invent a method for conjugation of PSs to carrier proteins by introducing hydrazide (-NH-NH_2) groups via hydrazine, ADH or carbohydrazide onto the protein molecules in the presence of lysine. The activated protein is then reacted with activated polysaccharide containing either aldehyde or cynate groups. Alternatively, we can introduce hydrazide (-NH-NH_2) groups onto a PS and then react them with activated proteins containing aldehyde groups. Because of the higher reactivity of hydrazide groups (pKa = 2.6) [19] compared to the amino group at neutral to mild acidic (pH 5.5-7.4) conditions, and the enhanced solubility of the conjugate using protein activation in presence of the amino acid lysine, the yield of the conjugation reaction is greatly increased. Due to the high yield, the leftover unconjugated PSs and proteins are unsubstantial, and the conjugate purification processes can be simplified by use of diafiltration to remove small molecules. We have shown that conjugates prepared by these methods are immunogenic in experimental animals (mouse). In addition, the conjugation reaction can be efficiently carried out under mild acidic or neutral pH at room temperature or at 4°C over night as opposed to days for the conventional methods. This again ensures high yield conjugate vaccine production for unstable polysaccharides such as those from *Haemophilus influenzae* type b, *Streptococcus pneumoniae* type 19F and *Neisseria meningitides* group A. The higher yield of our conjugation method compared to the conventional method is shown in Figure 3. Therefore, this invention will lead to production of less expensive conjugate vaccines and greatly promote public health.

MATERIALS AND METHODS

Materials

Tetanus toxoid (TT) was purchased from Lederle Vaccines, Pearl River NY and Wyeth Vaccines, Pearl River, NY. Meningococcal groups A and C polysaccharides (Mn A PS and Mn C PS, respectively) were from Bio-Manguinhos, Rio de Janeiro, Brazil. Pneumococcal (Pn) serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F were from Lederle Vaccines. Hydrazine, carbohydrazide, adipic acid dihydrazide (ADH), acetic hydrazide, 1-[3-(dimethylamino) propyl]-3-ethyl carbodiimide hydrochloride (EDC), sodium periodate, sodium borohydride, sodium cynoborohydride, 4-cyno-dimethylamino pyridium tetrafluoroborate (CDAP), and 1-amino-2, 3-propanediol were purchased from Sigma/Aldrich Chemical Company. TNBSA (2, 4, 6-trinitrobenzenesulfonic acid) and BCA (bicinchononoc acid) assay kit were purchased from Pierce.

Methods - This invention includes three hydrazide reactions (methods) for conjugating polysaccharides to protein described below using various PSs and tetanus toxoid as examples (see Table 3).

General Method A – Aldehyde-activated PS to hydrazide-activated protein

1. Hydrazide-activated TT was reacted with aldehyde-activated polysaccharide at ratio from 1:1 to 1:2 and concentration range 1-25 mg/mL in the presence of 3-15 mM sodium cyanoborohydride overnight, pH 5.5-7.4, 4-40°C.
2. $NaBH_4$ (twice moles of the aldehyde groups in the initial reactant) was then added for 3 hrs to reduce the unreacted aldehyde groups to alcohol.
3. The solution was dialyzed against saline adjusted to pH 7.4-7.6.
4. Total protein and polysaccharide contents in the conjugate product are essentially the same as the starting amounts of the reaction. They can also be determined by HPSEC for TT, resorcinol assay for meningococcal groups A and C polysaccharides, and anthrone assay for pneumococcal polysaccharides.
5. The unconjugated TT was determined by HPSEC and the unconjugated meningococcal group C polysaccharide determined by C18 particle absorption plus HPSEC using aldehyde-activated meningococcal group C polysaccharide as a standard.

General Method B – Cyanate-activated PS to hydrazide-activated protein

1. At 4°C, 2 mg hydrazide-activated TT was reacted with 4 mg cyanate-activated polysaccharide in 3-12 mL reaction mixture, pH 5.5-7.4 with an addition of 1 mg (0.1-0.4 mL) cyanate-activated polysaccharide at 4 and 8 hours.
2. After overnight reaction, the mixture was incubated at room temperature for 3-6 hours and then dialyzed against PBS, pH 7.4 at 4°C.
3. Free and conjugated proteins were determined by HPSEC.
4. Total PS was determined by anthrone assay.

General Method C - Hydrazide-activated PS to aldehyde-activated protein

1. Aldehyde-activated TT (0.4-1.6 mg/mL) was reacted with hydrazide-activated Pn 9V (0.8-3.2 mg/mL) overnight in the presence of 5 mM $NaBH_3CN$ with gentle agitation.
2. $NaBH_4$ (twice moles of the aldehyde in the initial reactant) was then added for 3-6 hrs to reduce the unreacted aldehyde groups to alcohol.
3. The reaction mixture was dialyzed against PBS, pH 7.4 at 4°C.
4. Free and conjugated proteins were determined by HPSEC.
5. Total PS content was determined by anthrone assay.

Immunogenicity of meningococcal group A and C polysaccharide-protein conjugates in mice

Immunization of mice

Mice (NIH-Swiss; groups of 10) were immunized with 1 ug/dose of polysaccharide or polysaccharide-protein conjugate on days 0 and 14. Antisera were collected on day 28 and assayed by ELISA for antibody levels against polysaccharide.

ELISA assay

Immunolon plates (Dynatech) were coated with 100 uL coating solution containing polysaccharide admixed with methylated human serum albumin overnight. After washing three times with 150 uL washing buffer (PBS with 0.05% Tween 20, 0.02% NaN_3), 100 uL of astiserum samples and reference serum (with 3200 units/mL anti-polysaccharide antibody; duplicate) at a serial two-fold dilution starting from 1/200 (diluted with dilution buffer containing PBS, 4% new born calf serum, 0.02% NaN_3), was added to each well. After overnight incubation, the plates were washed three times and incubated with 100 uL goat anti-mouse IgG Fc conjugated with alkaline phosphate (1/3000 dilution in dilution buffer) for two hours. After washing (3 x 150 uL) the plates were incubated with 100 uL p-nitrophenyl phosphate (1 mg/mL in 1 M Tris, pH 9) for 30 minutes and the reaction was stopped by 50 uL 0.1 N NaOH. The ELISA readings were measured with a plate reader and the anti-polysaccharide antibody levels of the antiserum samples were calculated from the ELISA readings and the standard curve of the reference serum (with 3200 units/mL antibody) co-assayed in the same plate. The geometric mean of antibody level for each mouse group was calculated.

Immunogenicity of Mn C PS-TT conjugates prepared by Method A

Mn C PS-TT conjugate products were prepared by three methods, using hydrazine or adipic acid dihydrazide as a spacer, activated Mn C PS alone or mixed with activated Mn A PS, using an activated Mn C PS/activated TT ratio of 5 mg/mL to 5 mg/mL at room temperature. Only minor fraction of un-conjugated free protein remained, shown as a right shoulder of the 280nm profiles in Figure 4 (The elution of the HPSEC column shifted for reasons unknown). These conjugates were used to immunize groups of 10 mice with plain polysaccharide as a control at 1 ug polysaccharide/dose on days 0 and 14. The geometric means of the induced antibody levels

(units/mL) two weeks post 2nd injection are 16 (8, 34; 1 SD confidence interval) for control group and 2141 (1069, 4285), 4228 (2189, 8167), 1092 (655, 1820) and 3977 (2423, 6526) for the four conjugate groups, assuming 3200 units/mL for the reference serum (Table 4). The conjugates also induced high levels of bactericidal antibody relative to the purified high molecular weight Mn C PS (Figure 5).

Immunogenicity of Mn A PS-TT conjugates prepared by Method A

Mn A PS-TT conjugate products were prepared using hydrazine or adipic acid dihydrazide as a spacer, activated Mn A PS alone or mixed with activated Mn C PS, with an activated MAPS/activated TT ratio of 5 mg/mL to 5 mg/mL at room temperature. Four different conjugates together with Mn A PS control were used to immunize groups of 10 mice at 1 ug polysaccharide/dose on days 0 and 14. The geometric means of the induced antibody levels (units/mL) two weeks post 2nd injection are 21 (7, 61; 1 SD confidence interval) for control group and 3831 (1930, 7606), 3963 (1353, 11611), 3991 (2382, 6687) and 3614 (2097, 6229) for the four conjugate groups, assuming 3200 units/mL for the reference serum (Table 5).

Conjugation example for Method A - Meningococcus group C conjugate

Materials

Tetanus toxoid (TT) was purchased from Wyeth Vaccines, Pearl River, NY. Meningococcal groups C polysaccharide (Mn C PS) was obtained from Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, Brazil. Hydrazine, carbohydrazide, adipic acid dihydrazide (ADH), acetic hydrazide, 1-[3-(dimethylamino) propyl]-3-ethyl carbodiimide hydrochloride (EDC), sodium periodate, sodium borohydride, sodium cynoborohydride, 4-cyno-dimethylamino pyridium tetrafluoroborate (CDAP), 2-(N-morpholino)ethanesulfonic acid (MES) and 1-amino-2, 3-propanediol were purchased from Sigma/Aldrich Chemical Company. TNBSA (2, 4, 6-trinitrobenzenesulfonic acid) and BCA (bicinchononoc acid) assay kit were purchased from Pierce.

Activation of Mn C PS to contain aldehyde groups

1. Mn C PS (10 mg/mL) was reacted with 3 mM $NaIO_4$ at 4°C overnight, pH 6.5.
2. After quench of the leftover $NaIO_4$ with 3 mM glycerol, the sample was dialyzed against H_2O at 4°C.
3. The concentration of the resulting activated PS was determined by a modified resorcinol assay using N-acetyl neuraminic acid as the standard with a correction factor of Mn C PS/N-acetyl neuraminic acid = 1.104/1.
4. The aldehyde content of the activated PS was determined by BCA assay using glucose as a standard.

The degree of activation of the activated Mn C PS prepared by this protocol is one aldehyde group per 79 monomers or 1.077 mg of aldehyde groups (mol. weight, 29) per gram Mn C PS (mol. weight of monomer, 341).

Activation of TT to contain hydrazide groups

1. TT (4 mg/mL) was reacted 60 minutes at room temperature with 0.36 M hydrazine in the presence of 12 mM EDC and 72 mM lysine at pH 6.
2. The reaction mixture was dialyzed against saline, pH 7.4 at 4°C.
3. The protein concentration of the resulting TT-hydrazide sample was determined by Lowry assay using bovine serum albumin as a standard.
4. The hydrazide content was determined by TNBSA assay using adipic acid dihydrazide as a standard.

The degree of activation of TT prepared by this protocol is 22 hydrazide groups per TT molecule, or 4.55 mg hydrazide group (mol. weight, 31) per gram TT (mol. weight, 150,000).

Conjugation of activated Mn C PS to activated TT

1. Hydrazide-containing TT (25 mg/mL), lyophilized and dissolved in water was reacted overnight at 40°C with aldehyde-containing Mn C PS (25mg/mL) in the presence of 10 mM sodium cyanoborohydride and 10 mM MES, pH 5.4.
2. The reaction mixture was treated with 2 mM $NaBH_4$ for 3 hours and then dialyzed against saline, pH 7.4.
3. Total protein was determined by Lowry assay using bovine serum albumin as a standard.
4. Total Mn C PS content was determined by a modified resorcinol assay using N-acetyl neuraminic acid as a standard.

The unconjugated free Mn C PS was determined by the method of C18 particle absorption of protein in the conjugate product followed by comparing the saccharide signal of the supernatant in HPSEC to those of the activated Mn C PS of known concentrations (Figures 1 and 2).

To estimate the yield of the yield of the conjugation, the conjugate product was diluted to approximately 1 mg/mL concentration of Mn C PS. One hundred microliter of this solution was mixed and incubated with 250 uL of activated C18 particles for an hour with gentle agitation. The supernatant was collected after centrifugation, and the C18 gel was washed twice with 100 uL saline. The combined the supernatant and wash was adjusted to 333 uL with saline and passed through a 0.2 um membrane microfilter. The filtrate was analyzed with HPSEC together with standard concentrations of activated Mn C PS at 0.033, 0.067 and 0.134 mg/mL, giving the area of the saccharide signals of these samples as 19.4, 4.8, 9.2, and 18.4, respectively. The saccharide concentration of the filtrate was calculated from the standard curve as 0.141 mg/mL, which was 3.3 times volume of the starting sample. Thus the starting sample contains 0.465 mg/mL (0.141 mg/mL x 3.3) free Mn C PS. The total Mn C PS concentration was determined as 1.131 mg/mL by modified resorcinol assay. The yield was estimated to be 59% (100% x (1-0.465/1.131)).

Physico-chemical assays of the reactants, activated PS and conjugate products

High performance liquid size-exclusion chromatography (HPSEC)

Samples of proteins, polysaccharides and conjugate products (25 uL, 0.1-1 mg/mL) were run through a Waters Ultrahydrogel 2000 column with saline at 0.5 mL/minute in a Dionex HPLC system with the Chromelean software and a UV detector at 280 and 206 nm. The UV detector at 280 monitors the signals of protein-containing species as well as compounds containing aromatic moieties and the signals of proteins, polysaccharides. The UV detector at 206 nm detects the protein and Mn C PS by presence of carbonyl groups, while the RI detector when used measures the signals of proteins, polysaccharides, conjugates and salts.

Discussion

Our conjugation chemistry and its improvements over the chemistry described by Shafer et al. and Lees et al. [20, 21]

In the method described by Shafer they link cyanate esters generated on the bacterial polysaccharide to ADH activated carrier protein using EDC [20]. By use of EDC the –COOH groups, principally on aspartic and glutamic acids, are converted to an unstable O-acetylisourea. The O-acetylisourea then reacts with ADH.

By our method the carrier protein is reacted with hydrazine dihydrochloride in presence of EDC and lysine, which leads to attachment of the hydrazide to the EDC activated –O-acetylisourea creating a hydrazone. This hydrazone is then reduced back to a stable, but reactive hydrazide, by use of sodium cyanoborohydride. The hydrazide activated protein is very stable. The hydrazide activated protein is easily conjugated to aldehyde groups or cyanate groups on the activated PS through a condensation reaction in the presence of sodium cyanoborohydride (to reduce the Schiff's base for the aldehyde condensation).

There are three major distinctions between our invention and the method described by Shafer/Lees. Firstly, in our invention, we use EDC to link hydrazine, carbohydrazide or adipic acid dihydrazide to protein in the presence of lysine to enhance the solubility of the protein-PS conjugate and to reduce its precipitation leading to a high yield of soluble product. Secondly, our invention covers not only the conjugation reaction of hydrazide groups on protein to cyanate groups on polysaccharide, but also the conjugation reaction of hydrazide groups on protein to aldehyde groups on polysaccharide and visa versa. Lastly, in our invention the conjugation reaction of hydrazide groups on protein to cyanate groups (Method B) on the polysaccharide does not use a blocking agent to block the unconjugated cyanate groups. We found that high concentration blocking reduces the yield of the conjugate product, because this conjugation reaction is reversible.

Cited references

1. Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrates. II. Immunological specificity of synthetic sugar-proteins. J Exp Med 1929; 50:533-550.

2. Schneerson R, Barrera O, Sutton A, Robbins JB. Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharide protein conjugates. J Exp Med 1980; 152:361-376.

3. Anderson P. Antibody responses to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugation of oligosaccharides of the type b capsule with the nontoxic protein CRM_{197}. Infect Immun 1983, 39:233-238.

4. Marburg S, Jorn D. Tolman RL, et al. Bimolecular chemistry of macromolecules: synthesis of bacterial polysaccharide conjugates with *Neisseria meningitidis* membrane protein. J Amer Chem Soc 1986; 108:5282-5287.

5. Kniskern PJ, Marburg S. Conjugation: design, chemistry, and analysis. In: Ellis RW, Granoff DM, editors. Development and clinical uses of Haemophilus b conjugate vaccines. New York: Marcel Dekker, 1994: 37-69.

6. Chu C, Schneerson R, Robbins JB, Rastogi SC. Further studies on the immunogenicity of Haemophilus influenzae type b and pneumococcal type 6B polysaccharide-protein conjugates. Infect Immun 1983; 40:245-256.

7. Schneerson R, Robbins JB, Parke JC, Bell C, Schlesselman JJ, Sutton A, Wang Z, Schiffman G, Karpas, A. Shiloach J. Quantitative analysis of serum antibodies elicited in adults by *Haemophilus influenzae* type b and pneumococcus type 6A capsular polysaccharide-tetanus toxoid conjugates. Inect Immun 1986, 52:519-528.

8. Anderson PW, Pichichero ME, Stein EC, Porcelli S, Betts RF, Connuck DM, Korones D, Insel RA, Zahradnik JM, Eby R.. Effect of oligosaccharide chain length, exposed terminal group, and hapten loading on the antibody response of human adults and infants to vaccines consisting of Haemophilus influenzae type b capsular antigen unterminally coupled to the diphtheria protein CRM197. J Immunol. 1989;142:2464-8

9. Jennings HJ, Lugowski C. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J Immunol 1981;127:1011-8.

10. Beuvery EC, Roy R, Kanhai V et al. Characteristics of two types of meningococcal group C polysaccharide conjugates using tetanus toxoid as protein carrier protein. Dev Biol Stand 1986;65:197-204.

11. Beuvery EC, Rossum FV, Nagel J. Comparison of the induction of immunoglobulin M and G antibodies in mice with purified pneumococcal type 3 and meningococcal group C polysaccharides and their protein conjugates. Infect Immun 1982;37:15-22.

12. Beuvery EC, Delft RV, Miedema F et al. Immunological evaluation of meningococcal group C polysaccharide-tetanus toxoid conjugate in mice. Infect Immun 1983;41:609-17.

13. Costantino P, Viti S, Podda A et al. Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C. Vaccine 1992;10:691-8.

14. Richmond P, Borrow R, Miller E et al. Meningococcal serogroup C conjugate vaccine is immunogenic in infancy and primes for memory. J Infect Dis 1999;179:1569-72.

15. Richmond P, Borrow R, Findlow J et al. Evaluation of de-O-acetylated meningococcal C polysaccharide-tetanus toxoid conjugate vaccine in infancy: Reactogenicity, immunogenicity, immunologic priming, and bactericidal activity against O-acetylated and de-O-acetylated serogroup C strains. Infect Immun 2001;69:2378-82.

16. Beuvery EC, Miedema F, Delft RV et al. Preparation and Immunochemical characterization of meningococcal group C polysaccharide-tetanus toxoid conjugates as a new generation of vaccines. Infect Immun 1983;40:39-45.

17. Beuvery EC, Miedema F, Delft RV et al. Vaccine potential of meningococcal group C polysaccharide-tetanus toxoid conjugate. J Infect 1983;6:247-55.

18. Guo Z, Jennings H. Protein-polysaccharide conjugation. In: Pollard AJ , Maiden MC (eds) Methods in Molecular Medicine, vol 66: Meningococcal Vaccines: methods and Protocols, Humana Press, Totowa, NJ, 2001, pg 49-54.

19. Inman JK, Dintzis HM. The derivatization of cross-linked polyacrylamide beads. Controlled introduction of functional groups for the preparation of special-purpose, biochemical adsorbents. Biochemistry 1969; 8:4074-4082.

20. Shafer DE, Toll B, Schuman RF, Nelson BL, Mond JJ, Lees A. Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) for use in protein-polysaccharide conjugate vaccines and immunological reagents. II. Selective crosslinking of proteins to CDAP-activated polysaccharides. Vaccine 2000; 18(13):1273-1281

21. Lees A, Nelson BL, Mond JJ. Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluroborate for use in protein-polysaccharide conjugate vaccines and immunological reagents. Vaccine 1996; 14:190-198.

Table 1. Physical-Chemical Comparison of Haemophilus b (Hib) Conjugate Vaccines

Vaccine	Saccharide size	Carrier protein	Spacer (linker)	Reference
PRP-D * (Connaught)	Polysaccharide	Diphtheria toxoid	6-carbon spacer (ADH)	2
HbOC (Wyeth-Lederle)	Oligosaccharide	Diphtheria protein (CRM)	None (amide)	3
PRP-OMPC (Merck)	Small polysaccharide	Meningococcal protein	Thioether (bigeneric)	4, 5
PRP-T (Aventis Pasteur)	polysaccharide	Tetanus toxoid	6-carbon spacer (ADH)	6, 7

* The four Hib conjugate vaccines are described commonly in the literature with these acronyms and the responsible manufacturers are in parentheses.

Table 2. Published conjugation methods that have been used to produce meningococcal conjugate vaccines

Method No	Saccharide size	Carrier protein	Spacer	Procedure	Used in humans	Reference
1. Reductive amination	Reduced	Tetanus toxoid	None	Aldehyde form of PS combined with protein in presence of cyanoborohydride	No	9, 10
2. Carbodiimide	Native	Tetanus toxoid	None	PS and protein combined in presence of carbodiimide, then blocked with ethanolamine	No	11, 12
3. Active ester [a]	Oligosaccharide	CRM $_{197}$	Adipic acid	Aminated reducing terminus of the oligosaccharide conjugated to protein by adipic acid $(NHS)_2$	Yes	13
4. Reductive amination	Reduced	CRM $_{197}$	None	Aldehyde form of saccharide combined with protein in presence of cyanoborohydride	Yes	14
5. Reductive amination	De-OAc PS [b]	Tetanus toxoid	None	Aldehyde form of PS combined with protein in presence of cyanoborohydride	Yes	9, 15

a Used N-hydroxysuccinimide diester of adipic acid
b. De – Acetylylated PS only reported for Meningococcal group C

Table 3. Conjugation methods employed for conjugating various polysaccharides from *Neisseria meningitides* (Mn), *Streptococcus pneumoniae* (Pn) and *Haemophilus influenzae* type b (Hib) to tetanus toxoid as a carrier protein.

Polysaccharides	Conjugation methods
Mn, group A	A, B
Mn, group C	A, B
Pn, type 1	A, B
Pn, type 3	A, B
Pn, type 4	A, B
Pn, type 6B	A, B
Pn, type 7F	A, B
Pn, type 9V	A, B, C
Pn, type 14	A, B
Pn, type 18C	A, B
Pn, type 19F	A, B
Pn, type 23F	A, B
Hib	A

Table 4. The geometric mean anti-Mn C PS antibody levels with 1 SD confidence interval of mouse groups (10 mice per group) two weeks post 2nd immunization with 1 ug/dose Mn C PS or each of four Mn C PS-TT conjugates prepared by conjugation method A.

Mouse groups of different immunogens	Geometric mean anti-MCPS antibody level, units/mL (CI)[a]
Mn C PS	16 (8, 34)
MCPS-TT (MC6xTTH)	2141 (1096, 4285)
MCPS-TT (MixTTHb)	4228 (2189, 8167)
MCPS-TT (MC6xTTADH)	1092 (655, 1820)
MCPS-TT (MixbTTADH)	3977 (2423, 6526)

a. Compared to a reference serum with anti-Mn C PS antibody level of 3200 units/mL.

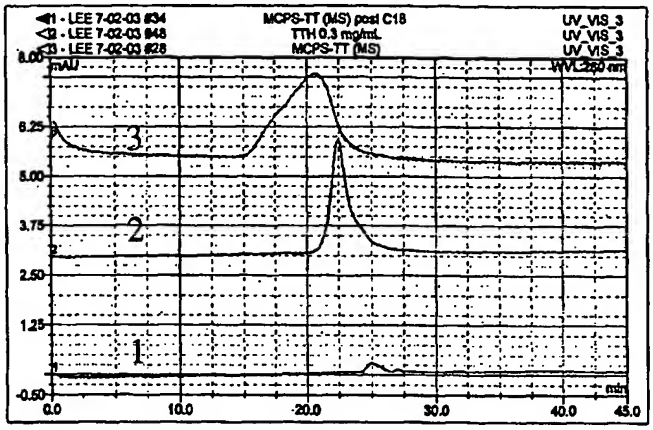
Table 5. The geometric mean anti-Mn A PS antibody levels with 1 SD confidence interval of mouse groups (10 mice per group) two weeks post 2nd immunization with 1 ug/dose MAPS or each of four MAPS-TT conjugates prepared by conjugation method A.

Mouse groups of different immunogens	Geometric mean anti-MAPS antibody level in units/mL (CI)[a]
Mn A PS	21 (7, 61)
MAPS-TT (MA4TTH)	3831 (1930, 7606)
MAPS-TT (MixTTHb)	3963 (1353, 11611)
MAPS-TT (MA6TTADH)	3991 (2382, 6687)
MAPS-TT (MixbTTADH)	3614 (2097, 6229)

a. Compared to a reference serum with anti-Mn A PS antibody level of 3200 units/mL.

Figure 1. Conjugation method A. conjugation of aldehyde groups on activated group C meningococcal polysaccharide to hydrazide groups on activated tetanus toxoid: Estimation of free polysaccharide

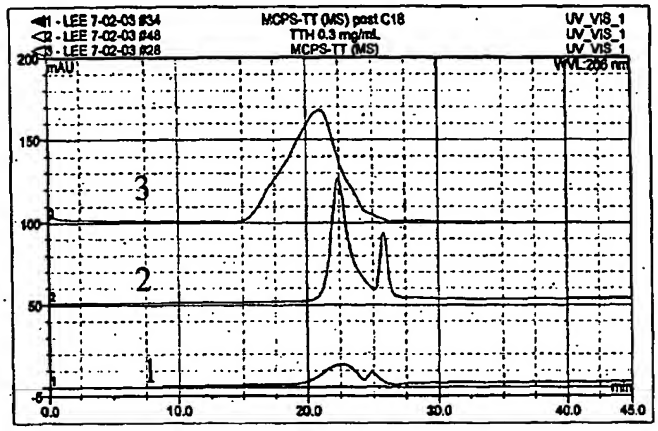
A



Absorption at 280 nm

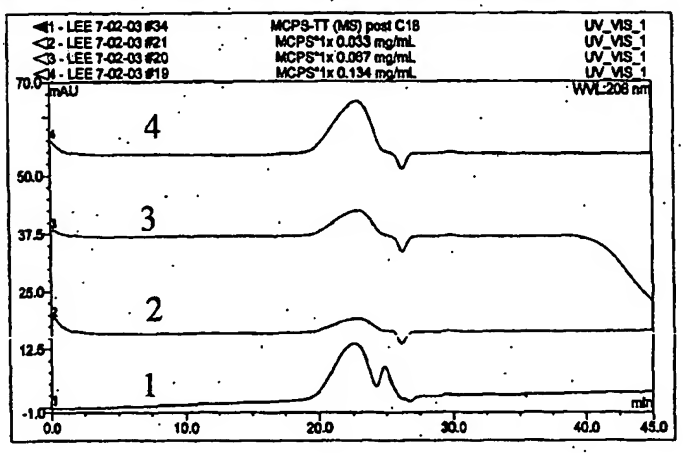
1. Conjugate post C18 absorb
2. Pure TT-H
3. Conjugate mixture before C18

B



Absorbence at 206 nm: Same three injections as as in A

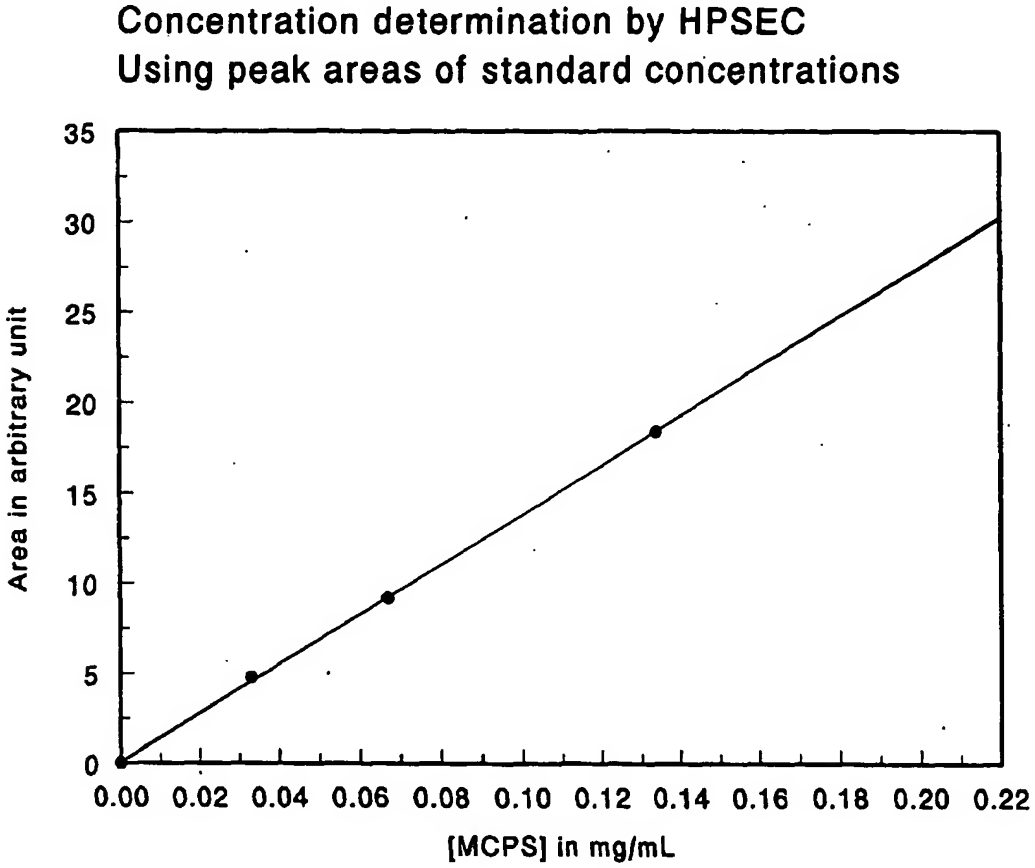
C



Absorbence at 206 nm: Comparison of free Mn C PS content of vaccine (1) with 0.033 mg/ml (2), 0.067 mg/ml (3), and 0.134 mg/ml of activated Mn C PS

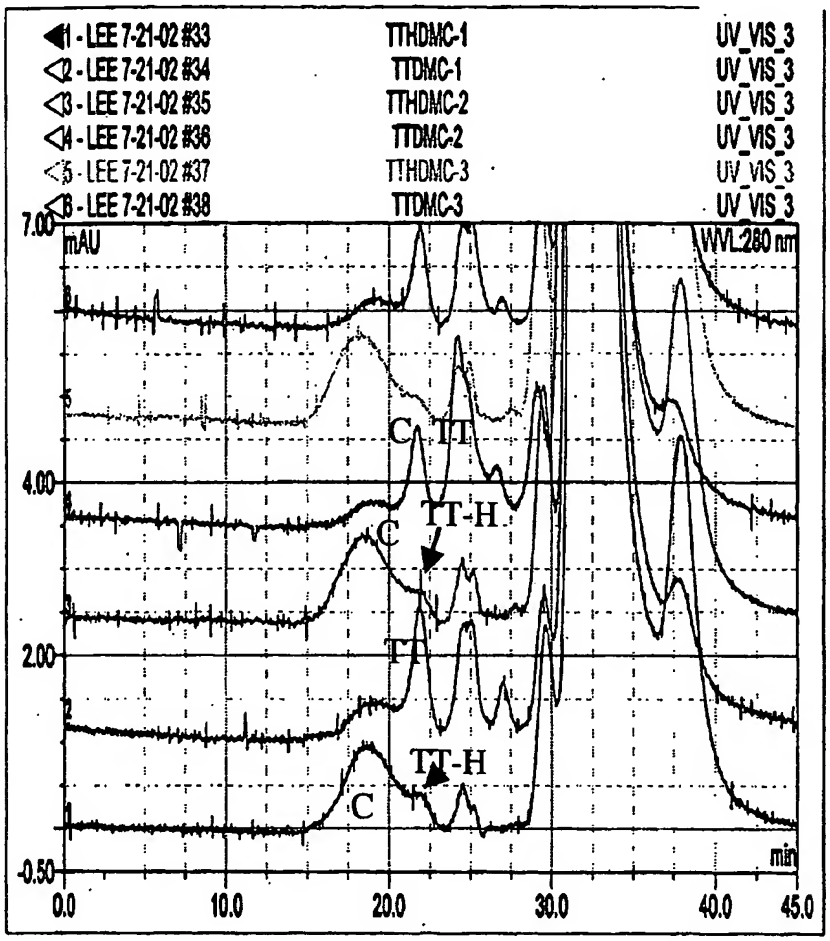
Figure 2

Quantitation of group C meningococcal polysaccharide in the conjugate product from Method A



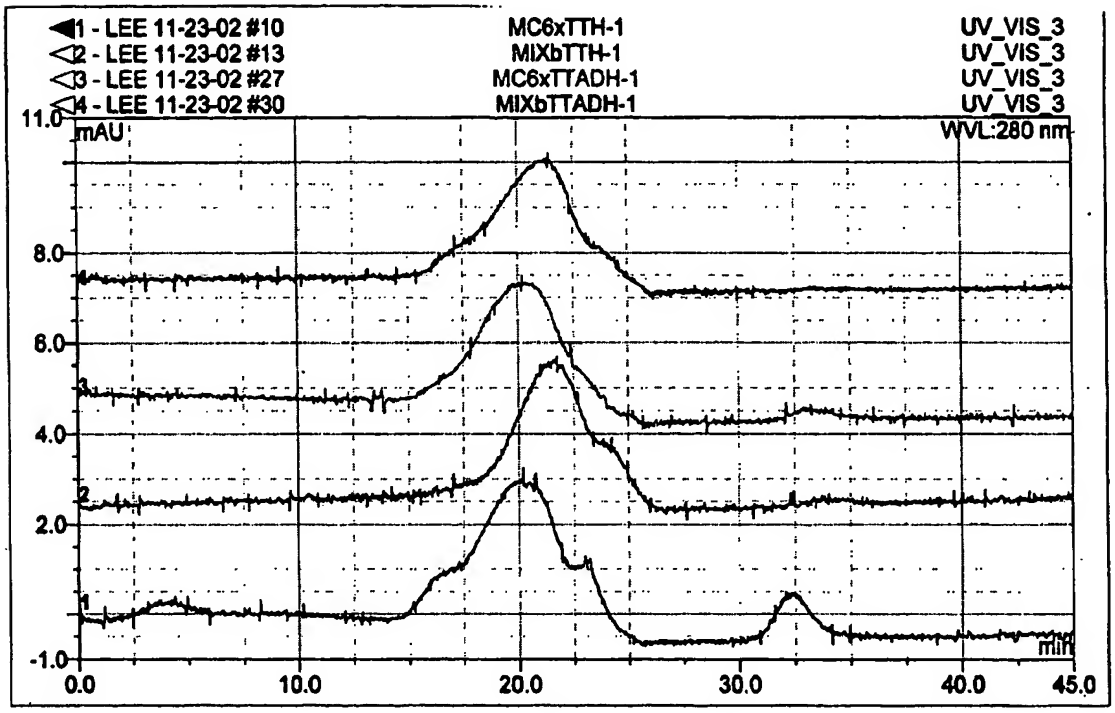
The residual size reduced polysaccharide was quantitated by comparison with a standard curve taken as the area under the signal for injection onto the SEC HPLC column of known activated (size reduced) group C meningococcal polysaccharide

Figure 3 Conjugation method A. Conjugation of aldehyde groups in activated group C meningococcal polysaccharide to hydrazide groups on activated tetanus toxoid

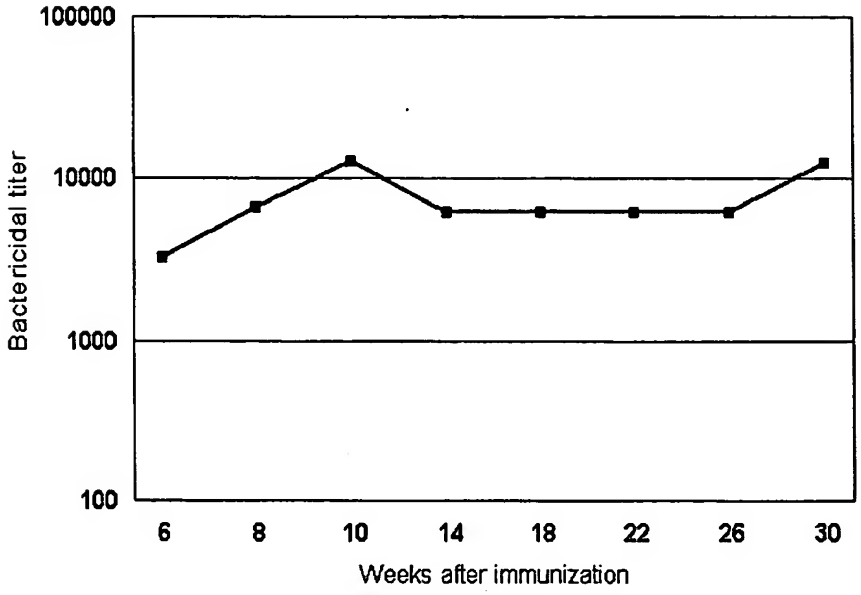


Three sets of conjugations comparing conjugation of periodate activated group C meningococcal polysaccharide a) amino groups on lysines(TT) [conventional method], and b) hydrazide groups on aspartic and glutamic acid residures (TT-H). Note that these spectra on conjugation products taken before the dialysis step and contain extra peaks at greater than 24 minutes not seen after dialysis..

Figure 4.
Meningococcal group C conjugates prepared by conjugation method a and used for immunization of mice



Figur 5. Bactericidal titers in mice immuniz d with the Mn C PS-TT conjugate vaccine



A group of five mice were immunized on weeks 0, 2 and 4 with 1 mcg of conjgate. The geometric mean titers are shown. A control group of 5 mice immunized with the Mn C PS had titers of < 100.

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/025477

International filing date: 06 August 2004 (06.08.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/493,389
Filing date: 06 August 2003 (06.08.2003)

Date of receipt at the International Bureau: 08 November 2004 (08.11.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse